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Award Number: W81XWH-05-1-0511

TITLE: Delphinidin: A Novel Agent for Inhibition of Breast Tumor Kinase Signaling by Targeting EGFR

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REPORT DATE: August 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE 01-08-2006		2. REPORT TYPE Annual		3. DATES COVERED 1 Aug 2005 – 31 Jul 2006	
4. TITLE AND SUBTITLE Delphinidin: A Novel Agent for Inhibition of Breast Tumor Kinase Signaling by Targeting EGFR				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-05-1-0511	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Farrukh Afaq, Ph.D.				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Wisconsin Madison WI 53706				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES Original contains colored plates: ALL DTIC reproductions will be in black and white.					
14. ABSTRACT Abnormalities in the expression and signaling pathways downstream of the epidermal growth factor receptor (EGFR) contribute to the progression, invasion, and maintenance of the malignant phenotype in breast cancer. EGFR is expressed at high levels in at least 25% of breast cancers and is associated with poor prognosis. Upon epidermal growth factor (EGF)-stimulation, breast tumor kinase (Brk) is recruited to the EGFR, and this event activates the catalytic activity of Brk, which in turn phosphorylates paxillin a binding partner and substrate for Brk. The phosphorylation of paxillin promotes the activation of Rac1, thereby stimulating cell migration and invasion in response to EGF. Many synthetic inhibitors of EGFR are known, but their use is limited because of their unacceptable cytotoxic effects on normal cells. Therefore, identification of a natural, nontoxic agent(s) as an inhibitor of EGFR is of utmost importance. Delphinidin, a major anthocyanin known to be present in pigmented fruits and vegetables, inhibits constitutive and EGF-induced phosphorylation of EGFR, activation of PI3K, phosphorylation of AKT, and MAPK. We also found that delphinidin treatment inhibits constitutive and EGF-induced activation of Brk signaling mediated through EGFR. Furthermore, treatment of breast cancer cells with delphinidin inhibited cell growth and induced apoptosis. In summary this study identifies an abundant fruits and vegetables based anthocyanin delphinidin as an effective blocker of EGFR signaling at least in breast cancer cells that act through novel Brk signaling pathway.					
15. SUBJECT TERMS Breast Cancer					
16. SECURITY CLASSIFICATION OF:			UU	18. NUMBER OF PAGES 15	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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INTRODUCTION:

The epidermal growth factor receptor (EGFR/HER1, also ErbB1) belongs to a family of four transmembrane receptor tyrosine kinases (EGFR/HER1, HER2, HER3, and HER4) that is activated in many epithelial tumors (1). Overexpression of EGFR occurs in about 25 % of human breast cancers and is associated with a poor clinical prognosis and therapeutic response (2). EGFR was the first tyrosine kinase receptor to be linked directly to human tumors (3). EGFR becomes biologically functional by forming a homodimer with itself or a heterodimer with other members of the receptor tyrosine kinases family. The mechanisms, which promote the formation of receptor dimers, include ligand binding and high receptor density due to overexpression. Several studies have shown that in many tumors epidermal growth factor (EGF)-related growth factors are produced either by the tumor cells themselves or are available from surrounding stromal cells, leading to constitutive activation of EGFR. The EGFR signaling pathway is involved in several cellular processes associated with malignant behavior, including cell proliferation, differentiation, apoptosis, migration, and cell adhesion. The signaling pathways induced by activated EGFR include the phosphatidylinositol 3-kinase (PI3K)/AKT and mitogen activated protein kinases (MAPK), both of which play a significant role in the mitogenic and cell survival responses mediated by EGFR. Abnormalities in the expression and signaling pathways downstream of the EGFR contribute to progression, invasion, and maintenance of the malignant phenotype in breast cancer (4).

Upon EGF-stimulation, breast tumor kinase (Brk/PTK6) is recruited to the EGFR, and this event activates the catalytic activity of Brk, which in turn phosphorylates paxillin a binding partner and substrate for Brk. The phosphorylation of paxillin promotes the activation of Rac1, thereby stimulating cell migration and invasion in response to EGF (5). Brk is a nonreceptor protein tyrosine kinase expressed in breast carcinoma tissue samples and breast tumor cell lines, and its expression is low or undetectable in normal mammary tissue or benign lesions (6). Brk expression sensitizes the mammary epithelial cells to the mitogenic response of EGF and potentiates their anchorage-independent growth (7). Accordingly, Brk association with EGFR enhances EGF-dependent phosphorylation of erbB3, which subsequently leads to an increased recruitment of PI3K and activation of Akt (8). EGFR is an upstream regulator of Brk signaling pathway through which Brk mediates EGF-induced phosphorylation of paxillin and activation of Akt, and involved in cell migration and invasion.

Many synthetic inhibitors of EGFR are known, but their use is limited because of their unacceptable cytotoxic effects on normal cells. Therefore, identification of a natural, nontoxic agent(s) as an inhibitor of EGFR is of utmost importance. Delphinidin, a major anthocyanin known to be present in pigmented fruits and vegetables (such as pomegranate, berries, dark grapes, egg plant, tomato, carrot and red onion) possesses potent antioxidant and antiproliferative properties. Therefore, modulating Brk's signaling with delphinidin by targeting EGFR may provide a promising cancer chemopreventive and cancer therapeutic target for breast cancer. In this study, employing human breast cancer cells AU-565 and non-tumorigenic human mammary epithelial cells MCF-10A that overexpress EGFR, we evaluated the effect of delphinidin on EGFR and its downstream signaling pathways. Through this study we envision identification of a natural, nontoxic dietary agent that can be exploited for the treatment of breast cancers that act through novel Brk signaling pathway by targeting EGFR.

BODY:

MATERIALS AND METHODS:

Materials: Delphinidin (>98% pure) was purchased from Extrasynthase (Lyon, France). The monoclonal and polyclonal antibodies for ERK1/2 (phospho-p44/42, Thr202/Tyr204), JNK (phospho-p54/46, Thr183/Tyr185), p38 (phospho-p38, Thr180/Tyr204), EGFR and phospho-EGFR at specific tyrosine sites 1068, 1045, and 845 were obtained from Cell Signaling Technology (Beverly, MA, USA). The antibodies for PI3K, phospho AKT, Rac1, Paxillin-phospho (Tyr118), Bcl-2, Bax and caspase-3 were procured from Upstate Biotechnology (Lake Placid, NY). Monoclonal antibody for PARP was purchased from Promega (Madison, WI, USA). The antibody for Brk was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The antibody for Paxillin-phospho (Tyr31) was procured from Chemicon International Inc. (Teleucula, CA, USA). Anti-mouse secondary horseradish peroxidase conjugate was obtained from Amersham Biosciences Limited (Buckinghamshire, England). Annexin-V-FLUOS staining kit was procured from Roche Diagnostic Corporation (Indianapolis, IN, USA). Antibiotic (Penicillin and Streptomycin) used were obtained from Cellgro Mediatech, Inc. (Herndon, VA, USA). Protein was estimated using BCA protein assay kit obtained from Pierce (Rockford, IL, USA).

Cell Culture: The AU-565 and MCF-10A cells were obtained from ATCC (Manassas, VA, USA). The cells were maintained under standard cell culture conditions at 37 °C and 5% CO₂ in a humid environment.

Treatment of Cells: Delphinidin (dissolved in DMSO) was used for the treatment of AU-565 and MCF-10A cells. The final concentration of DMSO used was 0.1% (v/v) for each treatment. For dose-dependent studies cells were treated with delphinidin (5-40 µM) for 3 and 48 hours in complete cell medium. Control cells were treated with vehicle alone. In additional experiments, serum starved AU-565 cells were treated with delphinidin (5-40 µM) for 3 hours and then incubated without or with EGF (50 ng/ml) for 15 min. In pilot experiments, it was established that DMSO (0.1% v/v) had no effects when measured at 48 h.

Cell Viability: The effect of delphinidin on the viability of cells was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) assay. The cells were plated at 1x10⁴ cells per well in 200 µl of complete culture medium containing 5, 10, 20 and 40 µM concentrations of delphinidin in 96-well microtiter plates for 48 h at 37 °C in a humidified chamber. Each concentration of delphinidin was repeated in 10 wells. After incubation for specified times at 37°C in a humidified incubator, MTT reagent (4 µL, 5 mg/mL in PBS) was added to each well and incubated for 2 hours. The microtiter plate containing the cells was centrifuged at 1,800 rpm for 5 minutes at 4°C. The MTT solution was removed from the wells by aspiration and the formazan crystals were dissolved in DMSO (150 µL). Absorbance was recorded on a microplate reader at 540 nm wavelength. The effect of delphinidin on growth inhibition was assessed as percentage inhibition in cell growth where vehicle-treated cells were taken as 100% viable.

Preparation of Cell Lysates and Western Blot Analysis: Following treatment of cells with delphinidin, the medium was aspirated and the cells were washed with PBS (10 mmol/L, pH 7.45). The cells were then incubated in ice cold lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1mmol/L EGTA, 1mmol/L EDTA, 20 mmol/L NaF, 100 mmol/L Na_3VO_4 , 0.5% NP-40, 1% Triton X-100, 1mmol/L phenyl methyl sulfonyl fluoride (PMSF) (pH 7.4), with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem, La Jolla, CA) over ice for 20 minutes. The cells were scraped and the lysate was collected in a microfuge tube and passed through a 21.5 G needle to break up the cell aggregates. The lysate was cleared by centrifugation at 14000 x g for 15 minutes at 4 °C, and the supernatant (total cell lysate) collected, aliquoted and was used on the day of preparation or immediately stored at -80 °C for use at a later time. For western blotting, 25-50 µg protein was resolved over 12 % polyacrylamide gels and transferred onto a nitrocellulose membrane. The non-specific sites on blots were blocked by incubating in blocking buffer (5% non fat dry milk/ 1% Tween 20 in 20 mmol/L TBS, pH 7.6) for 1 hour at room temperature, incubated with appropriate monoclonal primary antibody in blocking buffer for 90 minutes to overnight at 4 °C, followed by incubation with anti-mouse or anti-rabbit secondary antibody horse-radish peroxidase conjugate and detected by chemiluminescence and autoradiography using Hyperfilm obtained from Amersham Biosciences (UK Ltd.).

Detection of Apoptosis by Confocal Microscopy: The Annexin-V-FLUOS staining kit was used for the detection of apoptotic cells according to vendor's protocol. This kit uses a staining protocol in which the apoptotic are stained with annexin V (green fluorescence). MCF-10A and AU-565 cells were grown to about 60% confluence and then treated with delphinidin (5, 10, 20 and 40 µM) for 48 hours. The fluorescence was measured by a Zeiss 410 confocal microscope (Thornwood, NY). Confocal images of green annexin-FITC fluorescence were collected using 488 nm excitation light from an argon/krypton laser, a 560 nm dichroic mirror, and a 514–540 nm bandpass barrier filter.

RESULTS AND DISCUSSION:

Delphinidin treatment inhibits phosphorylation of EGFR in AU-565 and MCF-10A cells: We used western blot analysis to assess the effect of delphinidin in human breast cancer cells AU-565 and non-tumorigenic human mammary epithelial cells MCF-10A that overexpress EGFR. Using phosphospecific antibodies that detect specific tyrosine residues of EGFR at the 1068, 1045 and 845 sites, we found that the receptor was phosphorylated at all this residues in both the cell lines, although the level of phosphorylation differed in both the cell lines. Delphinidin (5-40 µM; 3 hours) treatment to EGFR positive cells AU-565 and MCF-10A resulted in a dose-dependent decrease in the phosphorylation of specific tyrosine residues of EGFR at 1068, 1045 and 845 sites (Figure 1A,B).

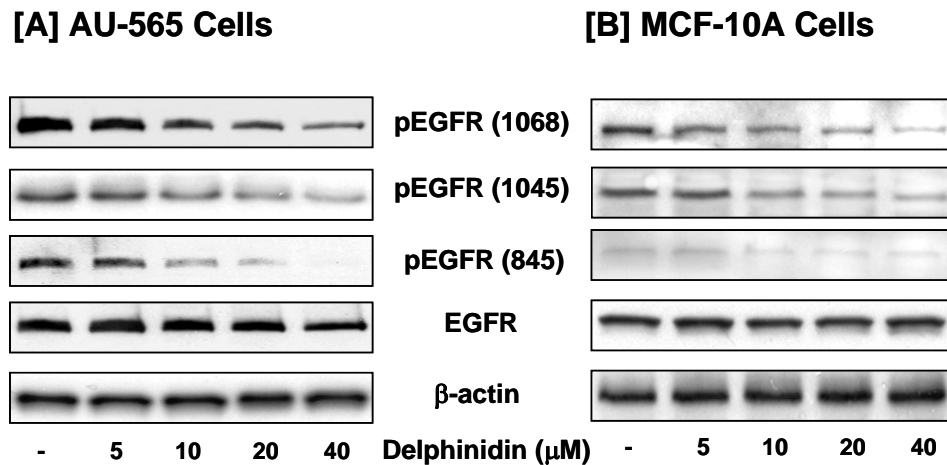


Figure 1: Effect of delphinidin on the phosphorylation of EGFR in AU-565 and MCF-10A cells. AU-565 and MCF-10A cells were treated with various concentrations of delphinidin (5–40 μ M) for 3 hours after which cells were harvested and total cell lysates were prepared for western blot analysis as detailed in 'Materials and methods'. Equal protein loading was confirmed by β -actin. The data shown here are from representative experiment repeated two times with similar results.

Delphinidin treatment inhibits protein expression of PI3K and phosphorylation of AKT and MAPK in AU-565 and MCF-10A cells: The signaling pathways induced by activated EGFR include the PI3K/AKT and MAPK, both of which play a significant role in the mitogenic and cell survival responses mediated by EGFR. We next evaluated the effect of delphinidin (5–40 μ M; 3 hours) treatment to EGFR positive cells AU-565 and MCF-10A on protein expression of PI3K and phosphorylation of AKT and MAPK. We found that delphinidin treatment inhibited protein expression of PI3K, phosphorylation of Akt at Ser⁴⁷³ and phosphorylation of MAPK (such as pERK1/2, pJNK1/2 and pp38) in a dose-dependent manner (Figure 2A,B).

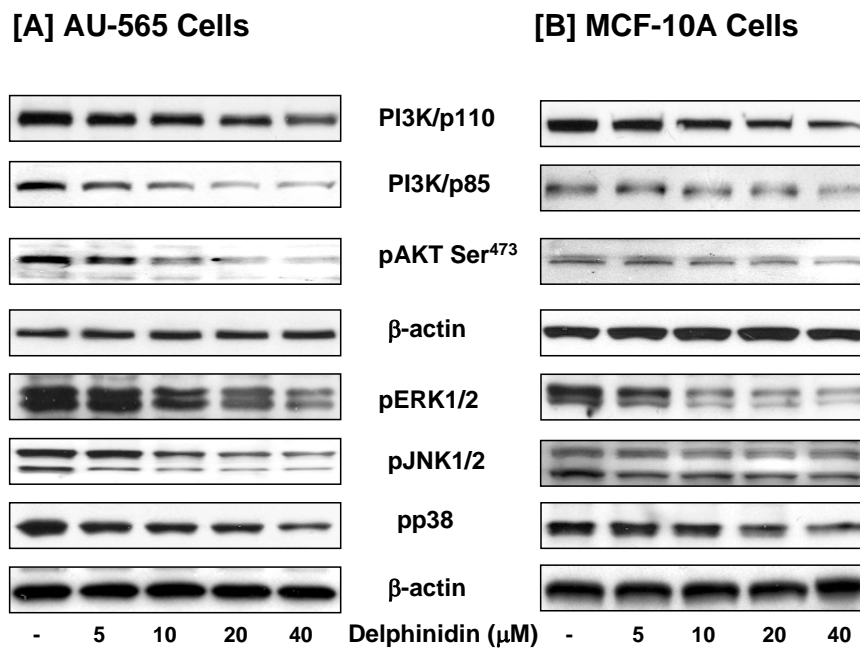


Figure 2: Effect of delphinidin on protein expression of PI3K and phosphorylation of AKT and MAPK in AU-565 and MCF-10 A cells. AU-565 and MCF-10A cells were treated with various concentrations of delphinidin (5–40 μ M) for 3 hours after which cells were harvested and total cell lysates were prepared for western blot analysis as detailed in 'Materials and methods'. Equal protein loading was confirmed by β -actin. The data shown here are from representative experiment repeated two times with similar results.

Delphinidin treatment inhibits EGF-induced phosphorylation of EGFR in AU-565 cells: In the next experiments we used western blot analysis to assess the effect of delphinidin on EGFR autophosphorylation by its ligand, EGF. AU565 cells, which express high levels of phospho-EGFR, were serum starved and then incubated with delphinidin (5-40 μ M) for 3 hours followed by a 15-minute incubation with EGF (50 ng/ml). Delphinidin treatment inhibits EGF-induced phosphorylation of EGFR at specific tyrosine residues 1068, 1045 and 845 (Figure 3). The total levels of EGFR remained unchanged. These results verified the ability of delphinidin to inhibit the activation of the EGFR.

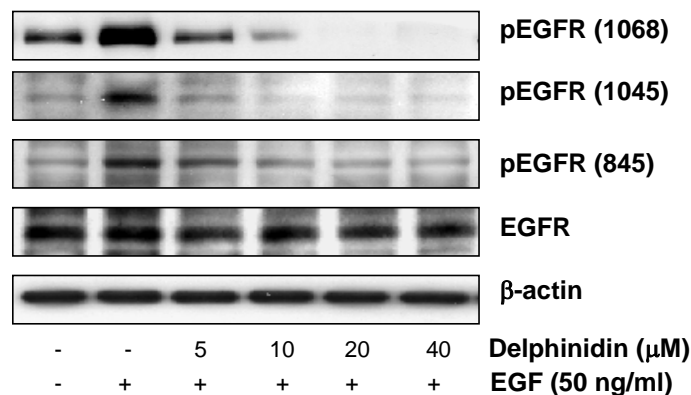


Figure 3: Effect of delphinidin on EGF-induced phosphorylation of EGFR in AU-565 cells. Serum-starved AU565 cells were treated with various concentrations of delphinidin (5–40 μ M) for 3 hours and then incubated without or with 50 ng/ml EGF for 15 minutes after which cells were harvested and total cell lysates were prepared for western blot analysis as detailed in 'Materials and methods'. Equal protein loading was confirmed by β -actin. The data shown here are from representative experiment repeated three times with similar results.

Delphinidin treatment inhibits EGF-induced activation of PI3K and phosphorylation of AKT and MAPK in AU-565 cells: Based on its ability to inhibit EGF-induced activation of the EGFR, we evaluated the effect of delphinidin treatment on the signaling cascades triggered by this receptor as well. The signaling pathways induced by activated EGFR include the MAPK and the PI3K/AKT pathways, both of which play a significant role in the mitogenic and cell survival responses mediated by this receptor. We found that delphinidin treatment inhibits EGF-induced activation of PI3K in a dose-dependent manner (Figure 4). The phosphorylation of Akt at Ser⁴⁷³ was almost completely inhibited at 20 μ M dose of delphinidin and the phosphorylation of MAPK were successfully inhibited at 10-20 μ M doses of delphinidin (Figure 4).

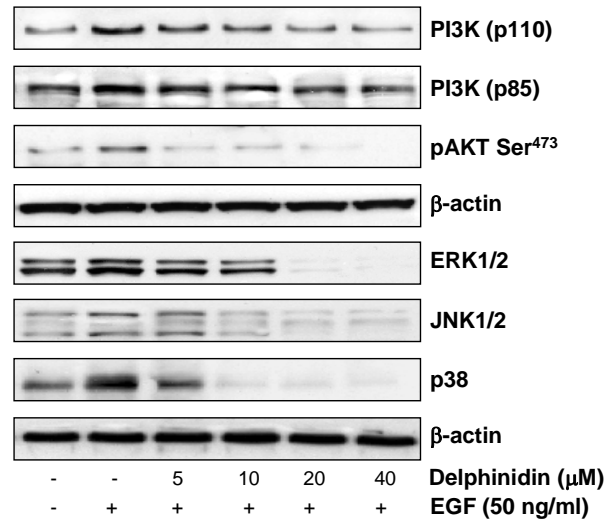


Figure 4: Effect of delphinidin on EGF-induced activation of PI3K and phosphorylation of AKT and MAPK in AU-565 cells. Serum-starved AU565 cells were treated with various concentrations of delphinidin (5–40 μM) for 3 hours and then incubated without or with 50 ng/ml EGF for 15 minutes after which cells were harvested and total cell lysates were prepared for western blot analysis as detailed in 'Materials and methods'. Equal protein loading was confirmed by β-actin. The data shown here are from representative experiment repeated three times with similar results.

Delphinidin treatment inhibits Brk protein expression and phosphorylation of paxillin and activation of Rac1 in AU-565 cells: We used western blot analysis to assess the effect of delphinidin in human breast cancer cells AU-565 that overexpress EGFR. Delphinidin treatment inhibits constitutive levels of Brk protein expression as well as phosphorylation of paxillin at Tyr³¹ and Tyr¹¹⁸ and activation of Rac 1 protein expression (Figure 5).

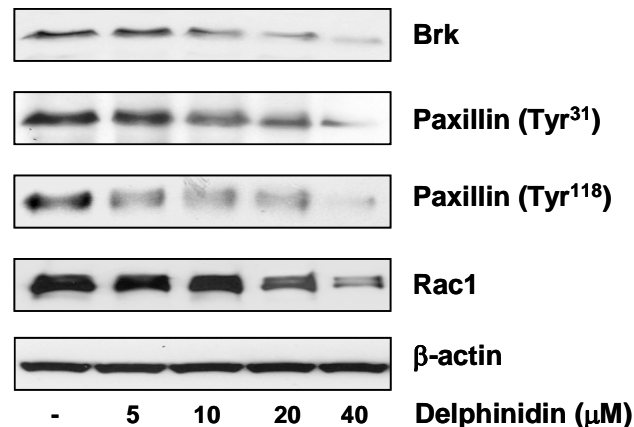


Figure 5: Effect of delphinidin on the levels of Brk protein expression and phosphorylation of paxillin and activation of Rac1 in AU-565 cells. AU-565 cells were treated with various

concentrations of delphinidin (5–40 μ M) for 3 hours after which cells were harvested and total cell lysates were prepared for western blot analysis as detailed in 'Materials and methods'. Equal protein loading was confirmed by β -actin. The data shown here are from representative experiment repeated two times with similar results.

Delphinidin treatment inhibits EGF-induced Brk protein expression and phosphorylation of paxillin and activation of Rac1 in AU-565 cells: Since EGF stimulation activates the catalytic activity of Brk, which in turn phosphorylates paxillin at Tyr³¹ and Tyr¹¹⁸. These phosphorylation events promote the activation of small GTPase Rac1 via the function of CrkII. Through this pathway, Brk is capable of promoting cell motility and invasion and functions as a mediator of EGF-induced migration and invasion. Our findings suggest that treatment of AU-565 cells with delphinidin inhibits EGF-induced expression of Brk and also inhibits phosphorylation of paxillin at Tyr³¹ and Tyr¹¹⁸ and activation of Rac 1 protein (Figure 6).

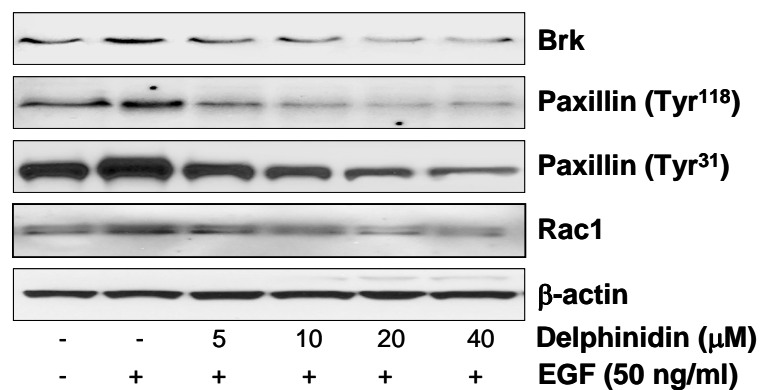


Figure 6: Effect of delphinidin on EGF-induced Brk protein expression and phosphorylation of paxillin and activation of Rac1 in AU-565 cells. Serum-starved AU565 cells were treated with various concentrations of delphinidin (5–40 μ M) for 3 hours and then incubated without or with 50 ng/ml EGF for 15 minutes after which cells were harvested and total cell lysates were prepared for western blot analysis as detailed in 'Materials and methods'. Equal protein loading was confirmed by β -actin. The data shown here are from representative experiment repeated three times with similar results.

Delphinidin treatment of AU-565 and MCF-10A cells results in growth inhibition: We next investigated whether delphinidin treatment imparts antiproliferative effects against AU-565 and MCF-10A cells. As shown in Figure 7, delphinidin treatment (5–40 μ M for 48 hours) of AU-565 and MCF-10A cells resulted in a dose-dependent inhibition of cell growth as assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide assay.

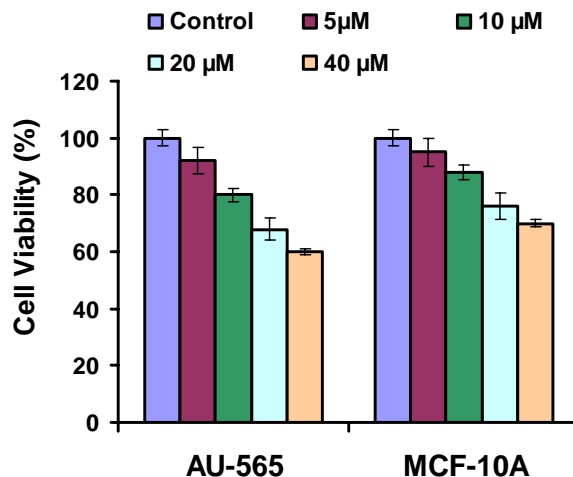
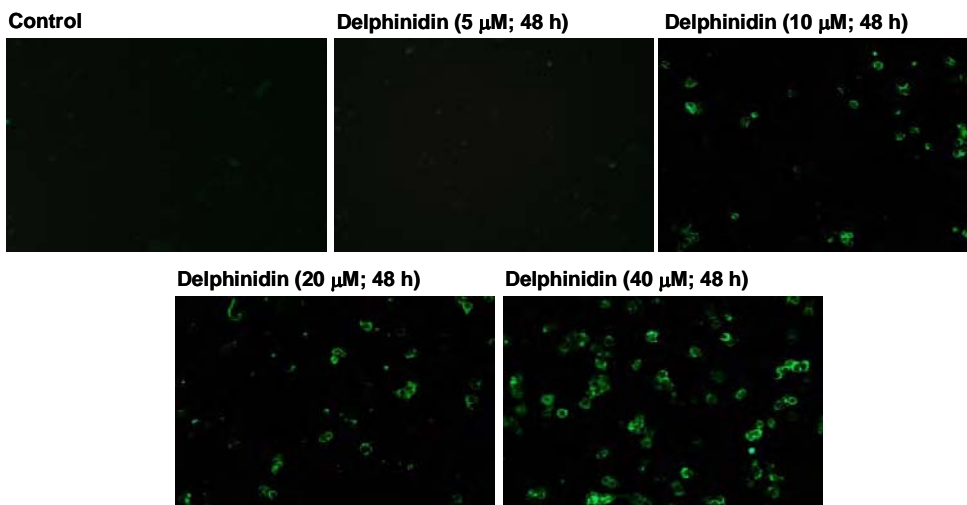


Figure 7: Effect of delphinidin treatment of AU-565 and MCF-10A cells on cell growth. The cells were treated with delphinidin (5-40 μ M) for 48 h, and the viability of cells was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide assay.

Delphinidin treatment of AU-565 and MCF-10 A cells results in apoptosis: To assess whether delphinidin-induced growth inhibition of the cells is mediated through induction of apoptosis. The extent of apoptosis was quantified by fluorescence microscopy of delphinidin (5-20 μ M; 48 hours) treated and untreated cells. As shown by the data delphinidin treatment resulted in induction of apoptosis in both AU-565 and MCF-10A cells as determined by fluorescence microscopy after staining the cells with Annexin V (Figure 8A,B). The effect of delphinidin was more pronounced in AU-565 cells.

[A] AU-565 Cells



[B] MCF-10A Cells

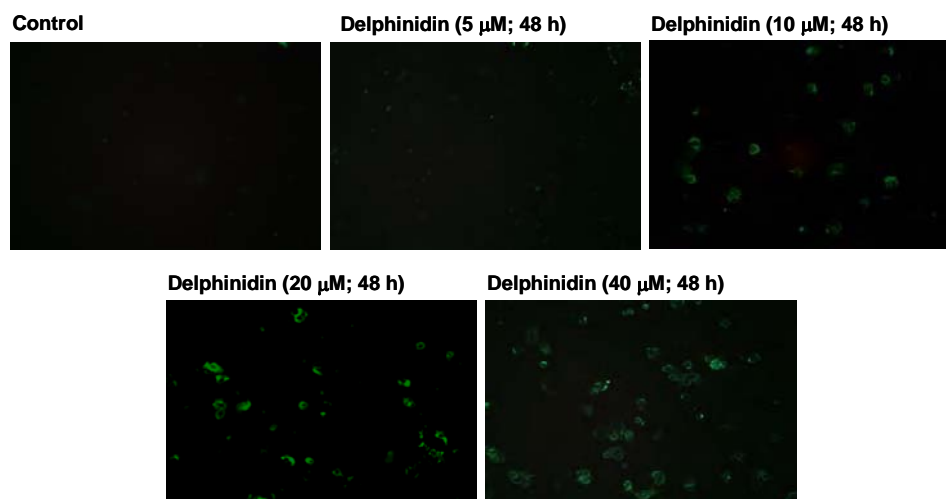


Figure 8: Delphinidin treatment of AU565 and MCF-10A cells results in apoptosis: The cells were treated with delphinidin (5-40 μ M) for 48 h, and the apoptosis was determined by fluorescence microscopy. The annexin-V-FLUOS staining kit was used for detection of apoptotic cells. The kit uses a staining protocol in which apoptotic cells are stained with annexin V (green fluorescence). A representative picture from two independent experiments with similar result is shown.

Delphinidin treatment of AU-565 and MCF-10A cells results in upregulation of Bax and downregulation of Bcl2: Because Bax and Bcl-2 play crucial roles in apoptosis, we next studied the effects of delphinidin on the protein levels of Bax and Bcl-2 in AU565 and MCF-10A cells. The immunoblot analysis exhibited an increase in the protein expression of Bax (Figure 9). In contrast, the protein expression of Bcl-2 was significantly decreased by delphinidin treatment in a dose-dependent fashion (Figure 9), thus further confirming the induction of apoptotic process.

[A] AU-565 Cells

[B] MCF-10A Cells

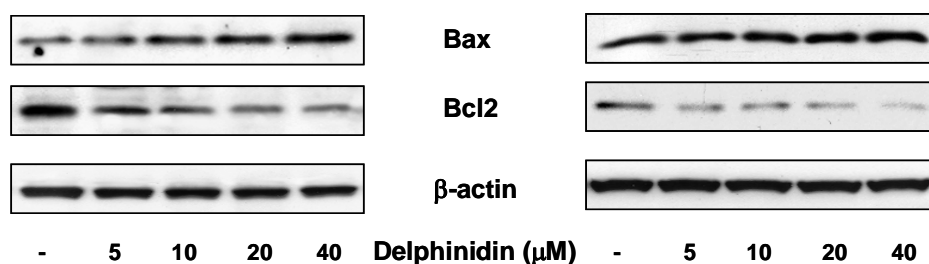


Figure 9: Effect of delphinidin treatment of AU-565 and MCF-10A cells on protein expression of Bax and Bcl2. The cells were treated with delphinidin (5-40 μ M) for 48 hours and cells were harvested, and cell lysates were prepared. The data are representative of three independent experiments with similar results. Equal protein loading was confirmed by β -actin.

The data shown here are from representative experiment repeated three times with similar results.

Delphinidin treatment of AU-565 and MCF-10A cells results in cleavage of PARP protein and activation of caspase 3. Next, we examined whether delphinidin treatment could induce poly(ADP-ribose) polymerase (PARP) cleavage. PARP is a 116 kDa protein that is cleaved into 85 kDa fragment during apoptotic cell death. As shown in Figure 10, employing immunoblot analysis, we found that the full-size PARP protein (116 kDa) was cleaved to yield an 85-kDa fragment after treatment of cells with delphinidin (5-40 μ M) for 48 hours. We next examined whether delphinidin treatment of AU-565 and MCF-10A cells resulted in activation of caspase 3. Our results suggest that treatment of cells with delphinidin (5-10 mM) resulted in a decrease in the protein expression of procaspase-3 in both the cell lines (Figure 10).

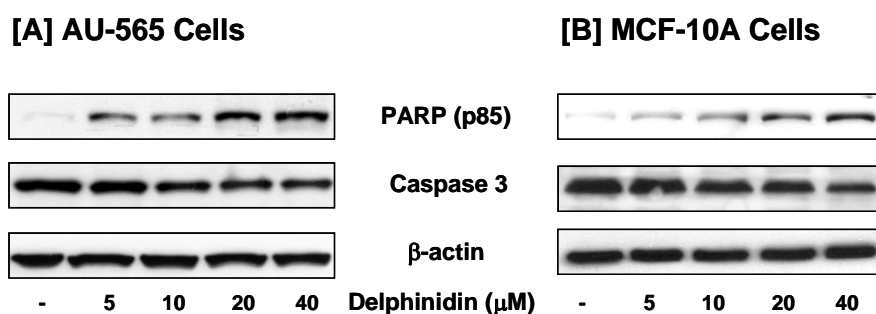


Figure 10: Effect of delphinidin treatment of AU-565 and MCF-10A cells on cleavage of PARP protein and activation of caspase 3. The cells were treated with delphinidin (5-40 μ M) for 48 hours and cells were harvested, and cell lysates were prepared. The data are representative of three independent experiments with similar results. Equal protein loading was confirmed by β -actin. The data shown here are from representative experiment repeated three times with similar results.

KEY RESEARCH ACCOMPLISHMENTS:

- Delphinidin, a major anthocyanin known to be present in pigmented fruits and vegetables, inhibits constitutive and EGF-induced phosphorylation of EGFR, activation of PI3K, phosphorylation of AKT and MAPK.
- Delphinidin treatment inhibits constitutive and EGF-induced activation of Brk signaling mediated through EGFR.

SIGNIFICANCE AND FUTURE STUDIES:

Abnormalities in the expression and signaling pathways downstream of the EGFR contribute to progression, invasion, and maintenance of the malignant phenotype in breast cancer. EGFR is expressed at high levels in at least 25 % of breast cancers and is associated with poor prognosis. Many synthetic inhibitors of EGFR are known, but their use is limited because of their unacceptable cytotoxic effects on normal cells. Therefore, identification of a natural, nontoxic

agent(s) as an inhibitor of EGFR is of utmost importance. Upon EGF-stimulation, Brk is recruited to the EGFR, and this event activates the catalytic activity of Brk, which in turn phosphorylates paxillin a binding partner and substrate for Brk. The phosphorylation of paxillin promotes the activation of Rac1, thereby stimulating cell migration and invasion in response to EGF. Delphinidin, a major anthocyanin known to be present in pigmented fruits and vegetables, inhibits constitutive and EGF-induced phosphorylation of EGFR, activation of PI3K, phosphorylation of AKT, and MAPK. We also found that delphinidin treatment inhibits constitutive and EGF-induced activation of Brk signaling mediated through EGFR. Furthermore, we found delphinidin inhibits Brk-induced paxillin phosphorylation and activation of Rac 1 signaling. Treatment of breast cancer cells with delphinidin inhibited cell growth and induced apoptosis. In summary this study identifies an abundant fruits and vegetables based anthocyanin delphinidin as an effective blocker of EGFR signaling at least in breast cancer cells that act through novel Brk signaling pathway.

Further studies are ongoing in normal cells to highly invasive cells including non-tumorigenic and tumorigenic cells to establish its therapeutic potential. The effect of delphinidin on the constitutive and EGF-induced activation of Brk's signaling mediated through EGFR by PI3K recruitment to erbB3 is in progress. We are also comparing the effectiveness of delphinidin with known synthetic inhibitors of EGFR on cell migration and invasion and to understand how the expression of Brk's signaling correlates with the EGFR expression.

REPORTABLE OUTCOMES:

Abstract

Zaman N, **Afaq F**, Khan N, Syed DN, and Mukhtar H. Delphinidin, a major anthocyanin in pigmented fruits and vegetables is a potent inhibitor of epidermal growth factor receptor and its downstream signaling pathway. The 97th Annual Meeting of the American Association for Cancer Research, Washington, DC, USA (April 1-5, 2006).

CONCLUSIONS:

This study identifies an abundant fruits and vegetables based anthocyanin delphinidin as an effective blocker of EGFR signaling at least in breast cancer cells that act through novel Brk signaling pathway.

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